RAPID, PCR-BASED METHOD FOR MEASURING TOTAL *ENTEROCOCCI* AND *BACTEROIDALES* IN WATER SAMPLES

A. SUMMARY

The following document describes a method for the detection of total *Enterococcus* and *Bacteroidales* in water samples based on the collection of these organisms on membrane filters, extraction of their total DNA, and polymerase chain reaction (PCR) amplification of group-specific DNA sequences using the TaqMan™ PCR product detection system and a real time PCR product detection instrument.

The TaqMan system signals the formation of PCR products by a process involving the nucleolytic degradation of a double-labeled fluorogenic probe that hybridizes to the target sequence at a site between the two PCR primer recognition sequences. The reactions are performed in a specially-designed thermal cycling instrument that automates the detection and quantitative measurement of the fluorescent signals produced by probe degradation during each cycle of amplification. These signals are stoichiometrically related to the quantities of PCR products produced. As is typical of PCR amplification, product formation initially follows a logarithmic curve, which subsequently falls off into a plateau. The ability to detect product formation during the initial logarithmic phase provides the basis for determinations of the starting quantities of target sequences in the reactions. During this phase, there is a log-linear relationship between these starting target sequence quantities and the number of amplification cycles required for the fluorescence signal to reach an instrument or user-defined threshold that is significantly above background. The instrument automatically calculates this cycle number for each reaction and reports it as a cycle threshold (C_T) value.

Procedures are further described for the use of C_T values generated by the instrument to enumerate target *Enterococcus* and *Bacteroidales* DNA and/or cell equivalents in the water samples. The approach described is based on the comparative cycle threshold (C_T) method (PE Biosystems, 1997), which employs an arithmetic formula to determine target sequence quantities in DNA extracts from test samples relative to those in similarly-prepared DNA extracts from calibrator samples containing a known quantity of target organism cells. C_T values for an exogenous control sequence or reference organism, added in equal quantities to both the test and calibrator samples before DNA extraction, are used to normalize results for differences in the amount of total DNA recovered from each sample (*e.g.*, caused by differences in DNA extraction efficiency between samples) and/or to signal potential PCR inhibition in test samples.

B. MATERIALS AND PROCEDURES

1.0 Preparation of glass bead extraction tubes **(performed by both Sampling and Analytical Labs)**:

(Note: it is recommended that preparation of these tubes be performed in advance of the water sampling and DNA extraction procedures described in sections 2, 3 and 4 below).

1.1 Materials:

1.1.1 Dedicated laminar hood or workstation for preparation of extraction tubes (Note: for the Analytical Lab, this can be the same hood or workstation as that is used for preparation of PCR reagents as described in section 6).

1.1.2 Weighing Balance (Note: can be outside hood but should be physically removed from any potential sources of DNA contamination).

1.1.3 Semi-conical, screwcap microcentrifuge tubes (PGC, #506-636), hereafter called extraction tubes.

1.1.4 Acid-washed glass beads (Sigma, # G-1277).

1.1.5 Autoclave

1.2 Procedures

Sampling Lab Note: For each sampling visit, prepare in advance a minimum of 1 extraction tube with glass beads for each water sample to be collected plus at least one additional extraction tube with glass beads to be used for filter blanks for each six water sample tubes. Tubes for multiple sampling visits can be prepared in advance.

Analytical Lab Note: For each batch of samples, prepare in advance a minimum of 6 extraction tubes with glass beads. Tubes for multiple analysis days can be prepared in advance.

1.2.1 Unscrew lids from extraction tubes and weigh 0.3 g $\pm$ 0.01 g of glass beads into each tube. Replace lids on the tubes and close tightly.

(Note: Make sure there are no glass beads on the O-rings of the caps that could stop the tubes from being sealed). Also visually check O-rings of the caps after closing to make sure they are seated properly.

1.2.2 Autoclave all extraction tubes with glass beads, prepared as described above, for 15 min at 121 PSI.

2.0 Water sample filtration **(Sampling Lab)**

2.1 Materials:

2.1.1 Dedicated workstation for water filtrations, preferably a laminar flow hood with UV light source.

2.1.2 Water filtration apparatus, including disposable or autoclaved filter funnels and bases for 47-mm filter; vacuum filter flask, or manifold and trap with appropriate tubing connected to vacuum source (line, aspirator or electric pump).

2.1.3 Autoclaved extraction tubes with glass beads, prepared as described in section 1.

2.1.4 Polycarbonate filters, 47-mm, 0.4-µm pore size (*e.g.,* Osmonics Inc., #K04CP04700).

2.1.5 PCR-grade water (e.g OmniPur water from VWR #EM-9610 or can be from in-house distillation or Milli-Q system if verified to be free of target DNA in negative control PCR reactions).

2.1.6 Two - Membrane filter forceps.

2.1.7 Ethanol, 95%.

2.1.8 Fisher or Bunsen burner.

2.1.9 Permanent marking pen for labeling tubes

2.2 Procedures:

(Note: this procedure can be performed simultaneously with multiple water samples depending on the type of filtration manifold in use. It is recommended that 1 negative control filter be prepared with each batch of 6 water samples (i.e. for each sampling visit) by the same procedure, omitting step 2.2.2. These negative control filters are referred to as filter blanks)

2.2.1 Remove funnel, place a polycarbonate filter on the filter base and replace funnel.

2.2.2 Shake the water sample bottle vigorously to suspend the bacteria uniformly. For surface water immediately measure 50-100 ml and pour it into the filter funnel. For waste water, similarly measure and filter 10-20 ml. Note: Selection of surface and waste water volumes to be filtered will be site-specific and dictated both by the volumes from that site that can be filtered in a reasonable amount of time and also by the requirement for adequate levels of target organisms in the filtered samples to allow their detection by the analytical method. Water volumes can be measured either by pipet, by graduated cylinder, or by graduations in the filter funnel. If

using filter funnel graduations, their accuracy should be confirmed against one or the other of the former volume measurement methods.

2.2.3 Apply vacuum to filter the sample, and rinse the sides of the funnel twice with 25 ml of PCR-grade water.

2.2.4 Turn off the vacuum and remove the funnel from the filter base.

2.2.5 Using sterile forceps, fold the filter on the filter base into a cylinder with the sample side facing inward, and insert filter into an extraction tube with glass beads, previously prepared as described in section 1.

(Note: Handle the filter with the forceps at the edges; do not touch the portion of the filter exposed to the water sample.)

2.2.6 Cap the extraction tube tightly and label.

2.2.7 Replace used filter funnels with new sterile funnels. Repeat steps 2.2.1 – 2.2.6 until all water samples have been processed.

3.0. Preparation and extraction of calibrator samples **(Analytical Lab)**.

3.1 Materials:

3.1.1 Dedicated laminar flow hood or workstation with UV light sources for work with DNA samples.

3.1.2 *Enterococcus faecalis*, American Type Culture Collection (ATCC) #29212, cell suspension: in 10 µl frozen aliquots in 1.5 ml microcentrifuge tubes. Note: cell suspensions and their estimated concentrations per ml will be provided by EPA. These cell suspensions may be supplied to the analytical lab in 100 µl aliquots. If so, it will be the responsibility of the analytical lab to sub-aliquot these suspensions.

3.1.3 *Bacteroides thetaiotaomicron*, (ATCC # 29741) cell suspension in 10 ul frozen aliquots in 1.5 ml microcentrifuge tubes. Note: cell suspensions and their estimated concentrations per ml will be provided by EPA. These cell suspensions may be supplied to the analytical lab in 100 µl aliquots. If so, it will be the responsibility of the analytical lab to sub-aliquot these suspensions.

3.1.4 Working stock of commercially available Salmon testes DNA (Sigma #D1626) dissolved in PCR grade water (e.g. OmniPur water from VWR

#EM-9610) at a concentration of ~10 ug/ml as determined by A260 reading in spectrophotometer)

3.1.5 AE buffer (Qiagen, #19077)

3.1.6 Polycarbonate filters, 47-mm, 0.4-µm pore size (*e.g.,* Osmonics Inc., #K04CP04700).

3.1.7 Extraction tubes with glass beads only, prepared as described in section 1.

3.1.8 Two membrane filter forceps.

3.1.9 Ethanol, 95%.

3.1.10 Fisher or Bunsen burner.

3.1.11 Single or 8-place mini bead beater (Biospec Corp., Bartlesville, OK).

3.1.12 Standard Microcentrifuge.

3.1.13 Vortex mixer.

3.1.14 Rainin Pipetmen: P-20, P-200 and P-1000 (or equivalent).

3.1.15 Rainin aerosol barrier pipet tips: 20, 200 and 1000 µl capacity (or equivalent).

3.1.16 1.7 ml low retention microcentrifuge tubes (e.g., #C-3228-1, GENE MATE)

3.1.17 Permanent marking pen for labeling tubes.

3.1.18 4^{o} C refrigerator.

3.1.19 -20^{o} or -70^{o} C freezer.

3.1.20 10% (v/v) bleach solution and autoclaved, distilled water for work station cleaning.

3.1.21 Disposable gloves

3.2 Procedures

(Note: To minimize chances of contamination of water sample filtrates and filter blanks, these procedures should be segregated from the filter

blank and water sample filtrate extraction procedures described in section 4 either by location or by time (e.g. at a different work station or on a different day of the week). However, they should employ the same batches of extraction buffer with salmon DNA that are used to prepare filter blank and water sample extracts as indicated in section 3.2.2. These materials can be stored at 4^{o} C to allow temporal segregation of the calibrator and water sample procedures if necessary.

3.2.1 With flame-sterilized forceps, fold six polycarbonate filters into cylinders and insert each into an extraction tube prepared as described in section 1.

3.2.2 Dilute Salmon DNA working stock with AE buffer to make 0.2 ug/ml salmon DNA/extraction buffer. Note: prepare sufficient salmon DNA/extraction buffer for the number of samples to be extracted in both sections 3 and 4 x 600 µl plus some extra (e.g. for 36 samples plus 6 sampling filter blanks, plus 6 calibrator samples, dilute 600 µl of 10 µg/ml working stock to 30 ml with AE buffer to make enough for 50 samples).

3.2.3 Remove one tube each of *E. faecalis* and *B. thetaiotaomicron* stock cell suspensions from freezer and allow to thaw completely.

3.2.4 Using Pipetmen with P-1000 & P-20 aerosol barrier tips, respectively, transfer 980 µl AE buffer to a tube containing 10 µl of *E. faecalis* stock cell suspension and mix thoroughly by vortexing. Transfer this suspension to tube containing 10 µl of *B. thetaiotaomicron* stock cell suspension and vortex again

3.2.5 Using a Pipetman with a P-20 aerosol barrier tip, spot 10 µl of the diluted and mixed *E. faecalis* and *B. thetaiotaomicron* cell suspension from step 3.2.4 on the inside surface of each filter cylinder from step 3.2.1. (Note: Vortex this cell suspension immediately before pipetting).

3.2.6 Using a Pipetman with a P-1000 aerosol barrier tip dispense 600 µl of Salmon DNA extraction buffer (prepared as described in 3.2.2) to each of the extraction tubes.

3.2.7 Cap the tubes as tightly as possible. Note: Visually inspect O-rings of the caps after closing to make sure they are seated properly.

3.2.8 Shake the extraction tubes on bead-beater for 60 sec. at maximum rate.

3.2.9 Centrifuge in microcentrifuge at 12,000 *x g* for 1 min to pellet glass beads and debris.

3.2.10 Using Pipetman and P-200 aerosol barrier tip, carefully transfer entire supernatants above glass beads from each sample to correspondingly-labeled, 1.7 ml, low retention microcentrifuge tubes.

Note: The filter will normally remain intact during the bead beating and centrifugation process. Collect as much of the supernatant as possible from around the filter .

3.2.11 Centrifuge supernatants at 12,000 *x g* for 5 min to pellet any remaining sediment.

3.2.12 Using a Pipetman with a P-200 aerosol barrier tip, transfer all but ~ 50 µl of clarified supernatants from second centrifugation to another set of clean, correspondingly-labeled, 1.7 ml, low retention microcentrifuge tubes. Note: take care not to disturb pellets. Inspect supernatant to confirm that it is clear (no turbidity).

3.2.13 Using a Pipetman with a P-200 aerosol barrier tip, transfer 40 µl of each clarified supernatant to another correspondingly labeled 1.7 ml, low retention microcentrifuge tube containing 160 µl of AE buffer (=5x dilution).

3.2.14 Refrigerate 5x-diluted samples for analysis the next day. For long term storage, freeze at -20^{o} or -70^{o} C. Store remainder of undiluted supernatants at -20^{o} or -70^{o} C for shipment to EPA lab.

3.2.15 When work is completed, treat all work surfaces in hood or work station with a 10% (v/v) bleach solution. Allow the bleach to contact the surfaces for at least 15 minutes prior to rinsing with autoclaved, distilled water. Turn UV lights on overnight.

4.0. Extraction of water sample filtrates and filter blanks **(Analytical Lab)**.

4.1 Materials:

4.1.1 Dedicated laminar flow hood or workstation with UV light sources for work with DNA samples..

4.1.2 Extraction buffer containing 0.2 ug/ml salmon DNA in AE buffer, prepared in section 3.2.2.

4.1.3 AE buffer (Qiagen, #19077)

4.1.4 Single or 8-place mini bead beater (Biospec Corp., Bartlesville, OK).

4.1.5 Standard Microcentrifuge.

4.1.6 Vortex mixer.

4.1.7 Rainin Pipetmen: P-20 and P-1000 (or equivalent).

4.1.8 Rainin aerosol barrier pipet tips: 20 and 1000 µl capacity (or equivalent).

4.1.9 1.7 ml, low retention microcentrifuge tubes (e.g., #C-3228-1, GENE MATE)

4.1.10 Permanent marking pen for labeling tubes

4.1.11 4^{o} C refrigerator

4.1.12 -20^{o} or -70^{o} C freezer.

4.1.13 Water sample filtrates and filter blanks (shipped from Sampling Lab)

4.1.14 10% (v/v) bleach solution and autoclaved, distilled water for workstation cleaning

4.1.15 Disposable gloves

4.1.16 Clean Lab Coat

4.2 Procedures

Note: Disposable gloves and clean lab coat should be worn for these procedures.

4.2.1 Using a Pipetman with a P-1000 aerosol barrier tip dispense 600 µl of extraction buffer containing 0.2 µg/ml Salmon DNA in AE buffer from section 3.2.2 to each filter blank & water sample filtrate extraction tube.

4.2.2 Close tubes as tightly as possible and label.(Note: Visually inspect O-rings of the caps after closing to make sure they are seated properly.

4.2.3 Shake the extraction tubes on bead-beater for 60 sec. at maximum rate.

4.2.4 Centrifuge in microcentrifuge at 12,000 *x g* for 1 min to pellet glass beads and debris.

Note: It is recommended that steps 4.2.5 – 4.2.8 be performed on filter blanks first and then on water sample filters.

4.2.5 Using Pipetman and P-200 aerosol barrier tip, carefully transfer entire supernatants above glass beads to correspondingly-labeled, 1.7 ml, low retention microcentrifuge tube. Note: The filter will normally remain intact during the bead beating and centrifugation process. Collect as much of the supernatant as possible from around the filter.

4.2.6 Centrifuge supernatants at 12,000 *x g* for 5 min to pellet any remaining sediment.

4.2.7 Using a Pipetman with a P-200 aerosol barrier tip, transfer all but ~ 50 µl of clarified supernatants from second centrifugation to another set of clean, correspondingly-labeled, 1.7 ml, low retention microcentrifuge tubes. Note: take care not to disturb pellets. Inspect supernatant to confirm that it is clear (may be colored but no turbidity)

4.2.8 Using a Pipetman with a P-20 aerosol barrier tip, transfer 40 µl of each supernatant to another correspondingly labeled 1.7 ml, low retention microcentrifuge tube containing 160 µl of AE buffer (=5x dilution).

4.2.9 Refrigerate 5x-diluted samples until ready for analysis as described in sections 5 & 6. For long term storage, freeze at -20° or -70° C. **Note: EPA analyses have indicated that DNA extracts obtained by the procedures described in this method may undergo significant loss of target sequences after long term storage either by refrigeration or freezing. It is therefore strongly recommended that all analyses of the DNA extracts be completed within 2-3 days of their preparation and no longer than within 1 work week.**

4.2.10 When work is completed, treat all work surfaces in hood or work station with a 10% (v/v) bleach solution. Allow the bleach to contact the surfaces for at least 15 minutes prior to rinsing with autoclaved, distilled water.

5.0 TaqMan Analysis **(Analytical Lab)**:

5.1 Materials:

5.1.1 At least two laminar flow hoods or workstations with UV light sources: #1] for preparation and aliquoting of PCR reagents to Smart Cycler reaction tubes (can be same hood or station as used for preparation of extraction tubes); #2] for additions of DNA extracts to reaction tubes (can

be same hood or station used for DNA extractions, however see sections 3.2.17 and 4.2.11)

5.1.2 Different protective clothing (labcoats) for each workstation.

5.1.3 10% bleach and autoclaved, distilled water for each workstation.

5.1.4 Disposable gloves separate box for each workstation.

5.1.5 TaqMan Universal PCR Master Mix (Applied Biosystems, #4304437).

5.1.6 Bovine serum albumen (BSA), fraction V powder.

5.1.7 PCR-grade water (OmniPur water from VWR #EM-9610)

5.1.8 *Enterococcus* forward primer: ECST748F;
5'-AGAAATTCCAAACGAACTTG, (Ludwig and Schleifer, 2000).

5.1.9 *Enterococcus* reverse primer: ENC854R;
5'-CAGTGCTCTACCTCCATCATT, (Ludwig and Schleifer, 2000).

5.1.10 *Enterococcus* TaqMan probe:GPL813TQ;
5'-(FAM)-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-3'(TAMRA or BHQ) (Ludwig and Schleifer, 2000).

5.1.11 *Bacteroidales* forward primer: GenBactF3;
5'-GGGGTTCTGAGAGGAAGGT (Siefring et al. 2007)

5.1.12 *Bacteroidales* reverse primer: GenBactR4;
5'-CCGTCATCCTTCACGCTACT (Dick and Field. 2004, Siefring et al. 2007.)

5.1.13 *Bacteroidales* probe: GenBactP2;
5'-CAATATTCCTCACTGCTGCCTCCCGTA-3'(TAMRA or BHQ), (Dick and Field. 2004, Siefring et al. 2007

5.1.14 Salmon DNA forward primer: SketaF2;
5'-GGTTTCCGCAGCTGGG (Haugland *et al.* 2005).

5.1.15 Salmon DNA reverse primer: SketaR2;
5'-CCGAGCCGTCCTGGTC (unpublished)

5.1.16 Salmon DNA reverse primer: SketaR3;
5'-CCGAGCCGTCCTGGTCTA (Haugland *et al.* 2005)

5.1.17 Salmon DNA TaqMan probe: SketaP2;

5'-FAM-AGTCGCAGGCGGCCACCGT-3'(TAMRA or BHQ) (Haugland *et al.* 2005)

5.1.18 1.7 ml, low retention microcentrifuge tubes (e.g., #C-3228-1, GENE MATE), separate container for each work station.

5.1.19 Test tube racks for 1.7-ml microcentrifuge tubes, separate racks for each work station .

5.1.20 Vortex mixer, one for each work station .

5.1.21 Standard microcentrifuge.

5.1.22 Smart Cycler 25 µl PCR reaction tubes (Cepheid, #900-0085).

5.1.23 Rack and microcentrifuge for Smart Cycler 25 µl PCR reaction tubes (provided with the instrument).

5.1.24 Rainin Pipetmen: P-10, P-20, P-200 and P-1000 (or equivalent), separate set for each work station.

5.1.25 Rainin aerosol barrier pipet tips: 10, 20, 200 and 1000 µl capacity (or equivalent), separate racks for each work station ..

5.1.26 Cepheid Smart Cycler® System

5.1.27 Printer (not mandatory)

5.1.28 Data archiving system (*e.g.*, ZIP drive and disks, LAN system connection to another computer, etc.).

5.1.29 *Enterococcus faecalis* and *Bacteroides. thetaiotaomicron* purified genomic DNA

Note: Genomic DNAs will be provided to the analytical laboratory with estimated target sequence copy concentration. An aliquot of this DNA stock should be serially diluted to make standards containing , 3.17×10^3, 1×10^3, 3.17×10^2, 1×10^2 , 3.17×10^1 and 1×10^1 target sequence copies/5 µl. **Freeze and save unused portion of this stock in case new dilutions are required in future**. Table 1 shows examples of dilutions needed to create these standards based on estimated concentration of stock provided being 4×10^5 target gene copies/5 µl. Standards should each be split into several replicate aliquots (note: work from lowest to highest concentrations to minimize cross-contamination). One set of aliquots should be stored at 4^o C for preliminary QC check and analysis with initial set of samples and remainder stored at -80^o C.

Table 1. Preparation of genomic DNA standards	*TSC/5ul	log TSC/5ul
Genomic DNA stock	4.00E+05	5.60
dilute 50 ul of DNA stock w/ 6250 ul AE (3x10e3 std)	3.17E+03	3.50
dilute 1750 ul of 3x10e3 std w/ 3800 ul AE (1x10e3 std)	1.00E+03	3.00
dilute 1750 ul of 1x10e3 std w/ 3800 ul AE (3x10e2 std)	3.17E+02	2.50
dilute 1750 ul of 3x10e2 std w/ 3800 ul AE (1x10e2 std)	1.00E+02	2.00
dilute 1750 ul of 1x10e2 std w/ 3800 ul AE (3x10e1 std)	3.17E+01	1.50
dilute 1750 ul of 3x10e1 std w/ 3800 ul AE (1x10e1 std)	1.00E+01	1.00
dilute 100 ul of 3x10e1 std w/ 900 ul AE (1x10e0 stample)	1.00E+00	0.00

* TSC = target sequence copies

Note: Preparation and analyses of 1x $10e^0$ samples are optional. These samples can be used to verify accuracy of target sequence copy concentrations in the standards. To do this analyze minimum of 20 – five ul aliquots of the 1x 10^0 sample with corresponding assay together with same number of reagent blank sample analyses. CT values of all positive analyses results should be within ~3 units of each other and at least 2 units lower than total # of cycles run (e.g. 43 if running 45 cycles). Assuming all reagent blank results are negative, percentage of negative results from the 1x 10^0 samples can be compared to a cumulative poisson distribution table with $\lambda = 1.0$. Accuracy of target sequence copy concentration estimates in the standards is verified if percentage of negative results is ~37%.

5.2 Procedures:

5.2.1 To minimize environmental contamination with amplified products, routinely treat all work surfaces with a 10% (v/v) bleach solution. Allow the bleach to contact the surface for at least 15 minutes prior to rinsing with autoclaved, distilled water. Use disposable gloves, and wear a separate clean lab coat at each workstation.

5.2.2 Using a Pipetman with aerosol barrier tips, add PCR grade water to the dried probe and primers from the vendor to create primary stock solutions of 100 µM probe and 500 µM primer, and dissolve by extensive vortexing. Centrifuge briefly. Note: Avoid strong lighting when working with probe stock solutions, store stock solutions at -20° C.

5.2.3 Prepare working stock solutions of probe/primer mixes for *Enterococcus, Bacteroidales* and two Salmon DNA assays indicated in Table 2 by adding 4 µl of probe stock and 10 µl of each primer stock to 576 µl PCR grade water, and vortex. Centrifuge briefly. Use a Pipetman with aerosol barrier tips for all liquid transfers. Store working stock solutions at -20° C. Note: It is recommended that these working stock solutions be aliquoted for storage into sufficient volumes for a single week's use.

Table 2. Primers and probes used in qPCR assays

Assay Name	target DNA	Forward Primer (section)	Reverse Primer (section)	Probe (section)
Entero1	*Enterococcus*	ECST748F (5.1.8)	ENC854R (5.1.9)	GPL813TQ (5.1.10)
GenBac3	*Bacteroidales*	GenBactF3 (5.1.11)	GenBactR4 (5.1.12)	GenBactP2 (5.1.13)
Sketa2	Salmon	SketaF2 (5.1.14)	SketaR3 (5.1.15)	SketaP2 (5.1.17)
Sketa22	Salmon	SketaF2 (5.1.14)	SketaR2 (5.1.16)	SketaP2 (5.1.17)

5.2.4 Using a Pipetman with aerosol barrier tips, prepare assay Master Mix working stocks of the *Enterococcus, Bacteroidales* and two Salmon DNA assay reactions in sterile labeled 1.6- ml microfuge tubes as follows:
- 2 µL of sterile H_2O/ reaction.
- 2.5 µL of BSA/reaction.
- 12.5 µL of TaqMan master mix/reaction.
- 3 µL of probe and primer working stock solution*/reaction.

[*This will give a final concentration of 1 µM of each primer and 80 nM of probe in the reactions]. Note: it is recommended that Master Mix working stocks be prepared at beginning of each day – see section 6.1. Preparing working stocks in advance and freezing is not recommended at this time because of uncertainty about the affects of freezing and thawing on the Taq polymerase enzyme.

5.2.5 Vortex the assay mix working stocks; then centrifuge briefly. Return unused primer/probe working stocks and the other reagents to the refrigerator.

5.2.6 Using a P-20 pipettor with aerosol barrier tips, add 20 µL of the assay mix working stocks to labeled 25 µl Smart Cycler tubes (Note: Try to avoid generating air bubbles because they may interfere with subsequent movement of the liquid into the lower reaction chamber.). The same tip can be used for pipetting multiple aliquots of the same assay mix as long as it doesn't make contact with anything else.

5.2.7 Cap the Smart Cycler tubes loosely and transfer to the appropriate PCR set-up station (see section 5.1.1).

5.2.8 Using a P-10 pipettor with aerosol barrier tips, add 5 µL of DNA extracts, appropriately diluted in AE buffer (see section 6) to corresponding labeled Smart Cycler tubes containing the assay mix working stocks then close the tubes tightly.

5.2.9 When all Smart Cycler tubes have been loaded, place them in a Smart Cycler centrifuge, and run for 2-4 sec.

5.2.10 Transfer the tubes to the Smart Cycler, inspecting each tube to verify that sample has properly filled the lower reaction chamber. (Note: A small concave meniscus may be visible at the top of the lower chamber, but no air bubbles should be present. If the lower chamber has not been properly filled, carefully open and reclose the tube, and recentrifuge.)

5.3 Smart Cycler Operation (Note: This protocol is intended to provide only information about critical instrument settings required to perform the EPA method. Further details concerning the operation of the instrument can be obtained from the Smart Cycler Operation Manual, Cepheid Part # D0190 Rev. D):

5.3.1 Turn on the Smart Cycler; then the computer.

5.3.2 Double-click on the **Smart Cycler** icon on the computer desktop.

5.3.3 The following steps for defining a protocol are only required before the initial run of the instrument. The protocol that is defined in these steps is used in all subsequent runs of the instrument.

5.3.3.1 Click on the **Define Protocols** icon to go to **Define Protocols** screen.

5.3.3.2 Click on the **New Protocol** button to open the **Protocol Name?** dialog. Enter "TaqMan 15-120" for the new protocol name, and click **OK**. The protocol stages are defined in the series of boxes at the bottom of the **Define Protocol** screen. To define **Stage 1**, click on its drop-down box to display the menu of stage types; then select **Hold**. In the **Temp** column, enter 50, and in **Secs** column, enter 120, leaving the **Optics** setting as the default **Off** setting. (Note: This stage is performed to eliminate potential PCR carryover products in the reactions using the Amp-Erase® UNG enzyme provided in the TaqMan Universal PCR Master Mix.).

5.3.3.3 To define **Stage 2**, click on its drop-down box to display the menu of stage types, and again select **Hold**. In the **Temp** column, enter 95, and in **Secs** column enter 600. Again, leave the **Optics** setting on **Off** (Note: This stage is performed to inactivate the Amp-Erase® UNG enzyme.).

5.3.3.4 To define **Stage 3**, select **2-Temperature Cycle** from its drop down menu. For the first step, enter 95 in the first row of the **Temp** column and 15 in the **Secs** column, **Optics** column **Off**. For the second step, enter 60 in the second row of the **Temp** column, 120 in the **Secs** column, and click on the **Optics** cell to select **On** from the drop-down menu. This sets the detection of the fluorescence signal to occur at the end of the second step in each cycle. Enter 45 in the **Repeat** field at the top of the **Stage 3** box to specify that it should be repeated for 45 cycles. Click the **Save Protocol** button.

5.3.3.5 To display primary curve graphs, click **Define Graphs**. Check the box for **Automatically add to new runs**. Under **Graph Type**

choose **Optics** from the pull down menu. Under **Channels** check the box for **Ch 1 (FAM)**. Under **Show** check the boxes for **Primary Curve** and **Threshold**. Under **Axes** check the box for **Fluorescence vs Cycle**.

5.3.4 Click on the **Create Run** icon to open the **Create Run** screen. For each new run, enter a unique name in the **Run Name** field. (Note: The software does not allow duplicate run names.).

5.3.5 Enter any additional information about the run in the **Notes** field. Click the arrow in the **Dye Set** box to display a drop-down menu of the possible selections. Select **FTTR25** (Note: This selects the dye set: FAM, TET, TAMRA, ROX, and a 25 µl reaction).

5.3.6 Click the **Add/Remove Sites** button. The **Select Protocols and Sites...** dialog will appear. Highlight (click on) the "TaqMan 15-120" protocol developed prior to the first run (See section 5.3.3) in the **Protocols** list. In the **Sites** list, highlight the sites on the instrument to be used with this protocol in the current run by clicking on them with the control key held down (Note: Sites refer to the I-core modules in the Smart Cycler processing block in which reaction tubes will be placed; a total of 16 are possible per block. When using multiple blocks, the site numbers will be preceded by the block letters, e.g. A, B C); then click the right pointing arrow to transfer the selected sites and protocol to the **Selections** table.

5.3.7 Click on the **OK** button to save the selections, and return to the **Create Run** screen.

5.3.8 Place the loaded Smart Cycler reaction tubes in the I-core module slots, selected above for current run. The tubes should snap into place. Either the front or back of the caps can face the front of the processing block.

5.3.9 In one of the **View** menus that is shown, select **Analysis Settings**. The displayed table includes one row for each of the four possible dye channels defined in the dye set. Click on the cell in the **FAM** row under the **Usage** column heading, and select **Assay** from the drop down menu. Set the **Usage** cells for all other dyes to **Unused** in the same manner. (Note: All assays in this protocol use FAM as the reporter dye.) All other cells in this table should be left at default settings (See Smart Cycler Operation Manual.). When finished editing the **Analysis Table**, click on the **Update Analysis** button.

5.3.10 In the other **View** menu that is shown, select the **Results Table**. Enter the sample identification information for each site in the **Sample ID** column (Additional information can be entered into the **Notes** column.). Leave

the other columns as default settings (See Smart Cycler Operation Manual.).

5.3.11 Click on the **Start Run** button. The orange LEDs on the Smart Cycler processing block should turn on, and the software will automatically switch to the **View Results** screen.

5.3.12 To display the real time temperature profiles for all sites, click **Temperature** in either of the **View** menus. To display real time growth curves for all samples (*i.e.*, the fluorescence signal vs. cycle), click **Primary** in the other **View** menu.

5.3.13 At the end of the run, it is recommended to check the cycle threshold values calculated by the instrument for each sample by opening the **Results Table** window by clicking on this selection in the upper **View** menu. It is also recommended to inspect the growth curves in the **Primary** window which can be opened in the same manner from the lower **View** menu. The default threshold fluorescence value is shown in this window as a single horizontal red line and the cycle thresholds for each site are shown as vertical red lines. To view the data for individual sites in this window, click on that site number in the table to the right of the graph. If the default threshold fluorescence line is well above all of the growth curve lines prior to visible amplification, the threshold fluorescence value can be changed to a lower value. This is done by reopening the **Analysis Settings** window from the upper **View** menu and entering a new value in the **Manual Thresh Fluor Units** cell in the **FAM** row. Conversely if the default threshold fluorescence line is below any of the growth curve lines prior to visible amplification, the threshold fluorescence value should be changed to a higher value in the same manner. Previous studies at EPA have indicated that a threshold value of 8 works well for most analyses. Other analytical labs should confirm that this threshold value is applicable for them (see section 6.1.4). Click on the **Update Analysis** button to view the new threshold line in the **Primary** window. The cycle threshold values will be automatically updated in the **Results Table**.

5.3.14 Once the threshold fluorescence value is adjusted to an optimal value, click the **Save Run** button. (Note: The Smart Cycler Software does not give a prompt to save changes before printing or exporting. Therefore, it is possible to make changes to the Results Table or Analysis Settings, and immediately print or export the data, then close the run without saving the changes. In this case, the data saved in the Smart Cycler database will not match the printed or exported data. If no changes are made in the threshold fluorescence value, the run data is automatically saved as it is when the program is closed or a new run is created).

5.3.15 To save the **Results Table and Analysis setting**, containing the instrument-calculated cycle threshold values for each sample, click the **Export** button to display the **Export Data** dialog box. Check the box next to the heading **Export Results Table and Analysis Settings** by clicking on it, while leaving all other boxes unchecked. Enter a path and a unique file name (Note: This can be same file name as the run file) by editing the area below the heading, **Export File Name:** (*e.g.*, export\filename.cvs), and then click on the **Export** button at the bottom of the window. Data are exported as comma-delimited text (**.cvs**) files in MS Excel-compatible files to the Export folder in the Smart Cycler folder: **C:\Program Files\Cepheid Smart Cycler 1.2\Export**.

5.3.16 To archive a run, close all other software programs, and select **Archive Run(s)** from **Database Menu**. Click **Proceed**. Select the run to be archived by clicking on its name in the database list. Select a folder, and enter an archive **Filename**. Click **Save**. The **Archive** dialog box will appear when the run has been successfully archived.

6.0 Sample Processing, Analysis and Data Quality Acceptance Guidelines **(Analytical Lab)**.

6.1 Sample Analysis

Note: Following guidelines assume that number of test samples may require analyses to be performed over several days each week. The first analyses each week should be to run reagent blanks, DNA standards and calibrator samples for the *Enterococcus* and *Bacteroidales* assays as described below. If number of test samples to be analyzed is small enough it is best practice to run reagent blanks, standards and calibrator samples each day prior to test samples.

6.1.1 Each week or day, determine the number of reactions to be performed for each assay and calculate volumes of master mixes for each assay needed as described in section 5. In dedicated work station #1 described in section 5, prepare master mix working stocks. Unused master mix working stocks can stored in refrigerator up to one week.

6.1.2 In workstation #1, set up 3 negative reagent control Entero1 and GenBac3 assay reactions by adding 5 µl AE buffer to Smart Cycler tubes containing 20 µl of respective master mixes.

6.1.3 In workstation #2, set up two reactions containing 5 ul of each *E. faecalis,* and *B. thetaiotaomicron* genomic DNA standard specified in section 5.1.29 and Entero1 and GenBac3 master mixes. Also set up two reactions containing 5 ul of 5x-diluted 0.2 ug/ml Salmon DNA extraction buffer

prepared for that week (section 3.2.2). Also set up one Entero1, GenBac3, Sketa2 and Sketa22 assay reaction each containing 5 ul of each 5x-diluted calibrator sample prepared that week.

6.1.4 Run negative reagent controls, standards and calibrator sample reactions in Smart Cycler blocks as described in section 5. At end of run, adjust fluorescence threshold as described in section 5.3.13 and inspect growth curves for each reaction to verify that C_T values are associated with true growth curves as opposed to spikes or drift in the fluorescence background. If latter instances are observed, re-adjust manual threshold values and update analysis as described in section 5.3.13. Check results for QC acceptance as described in section 6.2, save the run as described in section 5.3.14 and export the Results Table and Analysis settings as described in section 5.3.15.

6.1.5 Each day as needed set up Entero1, GenBac3, Sketa2 and Sketa22 assay reactions with filter blank and test sample extracts by adding 5 µl of each 5-fold diluted extract to one Smart Cycler tube containing 20 µl of each of the respective master mixes.

6.1.6 Run reactions prepared in 6.1.5 in Smart Cycler blocks as described in section 5.

6.1.7 At end of run adjust fluorescence threshold as described in section 5.3.13 and inspect growth curves for each reaction to verify that C_T values are associated with true growth curves as opposed to spikes or drift in the fluorescence background. If latter instances are observed, re-adjust manual threshold values and update analysis as described in section 5.3.13.

6.1.8 Save the run as described in section 5.3.14 and export the Results Table and Analysis settings as described in section 5.3.15.

6.1.9 Minimize the Smart Cycler window and open the newly-created export file from Windows Explorer (Note: the export file will be opened in the Excel spreadsheet program). Also open the Comparative C_T calculation template file in Excel. Copy the Entero1, GenBac3, Sketa2 and Sketa22 assay C_T values for the calibrator samples, test samples and negative controls and paste in the appropriate cells of the Comparative C_T calculation template file (Note: this file should already contain appropriate formulas entered for all calculations). Copy the avg. calibrator ΔC_T value calculated by the spreadsheet into the corresponding cells of each test sample row. The spreadsheet will then automatically calculate and report the estimated total number of *Enterococcus* and *Bacteroidales* cells as Calibrator Cell Equivalents (CCE's) in the original water sample for each test sample. Concentrations can be calculated by dividing this number by the volume of

water filtered. (Note: if another run of the Smart Cycler is to be performed immediately, copy the export file to a floppy disk or rewritable CD and transfer to another computer before doing these calculations. Cepheid, Inc. advises against opening additional programs while the Smart Cycler is in operation.)

6.2 Data Quality Acceptance

6.2.1 If greater than one-third of the reagent controls reactions with a particular master mix give positive signals (CT values below 45) or if any of the CT values are lower than 35, the analyses should be repeated with new Master Mix working stock preparations.

6.2.2 Results from genomic DNA standards reactions for each assay should be subjected to regression analysis of the $\log_{10}$–transformed target sequence copy numbers on CT values. The r-squared values for these regressions should be greater than 0.98 and the slope values should be consistent with laboratory's historical averages. If the r-squared values are acceptable (excluding a maximum of one obvious outlier CT value is permitted) but slope differences are greater than 0.3 units from these averages, the genomic standards should be reanalyzed. If the slope differences persist, new amplification factor values should be calculated from these slopes and these values should be applied in ΔCT and ΔΔCT calculations as described in section 7.

6.2.3 Mean CT values for the Sketa2 and Sketa22 assays of the 5x-diluted 0.2 ug/ml Salmon DNA extraction buffer prepared for that week should be within 3 CT units of the laboratory's historic average.

6.2.4 In general, target cell concentrations can be calculated from the results of the 5x-diluted samples as described in section 7 if their Sketa2 assay results are within 3 CT units of the mean results from the calibrator samples. Higher values may indicate PCR inhibition. Repeat *all* PCR reactions of any samples giving a Sketa2 DNA assay CT value greater than 3 CT units higher than the mean of the calibrator sample results using 5 µl of an additional 5x dilution of the already 5x-diluted extracts in AE buffer. Also repeat *all* PCR reactions of the calibrator samples after the same additional 5x dilution of the already 5x-diluted extracts in AE buffer. The result from the original 5x-diluted sample can be tentatively accepted (with a notation that it failed QC test) if its Sketa2 assay CT value is lower than that of the corresponding 25x-diluted sample (assumption here is that there is negligible inhibition but poor recovery of total DNA in the extract - this may be corrected for by the calculation method). If the CT value of the 25x-diluted sample is lower and passes QC criterion indicated above relative to calibrators analyzed at same 25x-dilution, then the result from

this sample is can be tentatively accepted (with a notation that it was analyzed at 25x-dilution).

7.0 Calculation of target cell numbers in test samples from TaqMan analysis Cycle Thresholds **(Analytical Lab)**.

TaqMan analysis permits simplified determinations of the ratios of the target sequences in a test sample compared to a calibrator sample using an arithmetic formula, referred to as the Comparative Cycle Threshold Method. These ratios can be converted to measurements of calibrator cell equivalents (CCE) in test samples through the use of calibrator samples containing a known quantity of the target organism cells.

7.1 Procedures

(note: all calculations, including data quality calculations described in section 6.2, should be performed in an Excel spreadsheet and all formulas should be maintained to indicate how final results were arrived at. A template Excel file may either be provided by EPA for this purpose or developed by the analytical lab and submitted to EPA in advance of study for approval)

7.1.1 $\Delta\Delta C_T$ calculation (example in Table 3)

7.1.1.1 Subtract the Sketa22 assay C_T value ($C_{T,ref}$) from the target assay C_T value ($C_{T,target}$) for each calibrator sample extract to obtain $\Delta C_{T,cal}$. Calculate average $\Delta C_{T,cal}$ for all calibrator samples**.(note: only Sketa22 assay results are henceforth to be used in these calculations. Sketa2 assay results are used only as a QC test to detect potential PCR inhibition as described in section 6.2.4).**

7.1.1.2 Subtract the Sketa22 assay C_T value from the target assay C_T value for each water sample extract to obtain $\Delta C_{T,test}$.

7.1.1.3 Subtract average $\Delta C_{T,cal}$ from $\Delta C_{T,test}$ to obtain $\Delta\Delta C_T$.

7.1.1.4 Calculate the ratio of the target sequences in the test and calibrator samples using the formula: F^(-ΔΔCT), where F = amplification factor of the target organism PCR assay. [Note: current amplification factor value observed at the USEPA laboratory in Cincinnati is 2.0, however, it is recommended that each analytical laboratory determine these values for their own instruments and reagents before beginning analyses of test samples by generating standard curves of assay CT values from serially diluted genomic DNA extracts of the target organisms and regression analysis of log

cell equivalents on CT. Amplification factors can be calculated from the formula: F = 10^(1 / -X coefficient of the regression)]

7.1.1.5 Multiply the ratio of the target sequences in the test and calibrator samples by the number of target organism cells in the calibrator sample to obtain estimated number of target organism cells (CCE) in the test sample. Note: following protocol in section 3, number of cells in calibrator samples can be calculated from EPA-provided estimates of cells/ml in provided cell suspensions (see sections 3.1.2 and 3.1.3) by dividing these numbers by 10,000.

7.1.2 ΔC_T calculation (example in Table 4)

7.1.2.1 Calculate average ($C_{T,target}$) for all calibrator samples

7.1.2.2 Subtract average $C_{T,cal}$ from $C_{T,test}$ for each test samples to obtain $\Delta C_{T,target.}$

7.1.2.3 Calculate the ratio of the target sequences in the test and calibrator samples using the formula: F^(-ΔCT), where F = amplification factor of the target organism PCR assay (see 7.1.1.4).

7.1.2.4 Multiply the ratio of the target sequences in the test and calibrator samples by the number of target organism cells in the calibrator sample to obtain estimated number of target organism cells (CCE) in the test sample. (see section 7.1.1.5).

7.1.3 Reanalyses

7.1.3.1 Test samples failing reference assay QC criteria should be reanalyzed as described in section 6.2.4. Calculations for the samples can be performed in same manner as described in sections 7.1.2 and 7.1.3. (Note: it is necessary to also reanalyze calibrator sample extracts with target assay at new dilution for ΔC_T calculation. While not essential for $\Delta\Delta C_T$ calculation, it is best practice to also reanalyze calibrator samples at new dilution).

7.1.4 Data Reduction

7.1.4.1 The geometric mean of the CCE and associated coefficients of variation in replicate test samples or samples from same sampling time can be determined from individual test sample C_T values using the following procedures:

7.1.4.1.1 Calculate the $\log_{10}$ of the measured CCE in each sample log(N).

7.1.4.1.2 Calculate the mean (M) and standard deviation (S) from the values of log(N) obtained in the previous step for the all of the replicate filter extracts or filter extracts from the sampling time.

7.1.4.1.3 Calculate the geometric mean as 10^{M}.

7.1.4.1.4 The implied coefficient of variation (CV) is calculated, based on the lognormal distribution, as the square root of $10^{V/\,0.434} - 1$, where $V = S^2$.

Table 3. Example $\Delta\Delta C_T$ Calculations (Amplification factor = 2):

Target Cells in Sample	Sample Type	$C_{T,target}$	$C_{T,ref}$	ΔC_T	$\Delta\Delta C_T$	CCE in Test Sample ($2^{-\Delta\Delta CT}$ x cells in Calib[1])
10000	Calib[1]	21.4	18.3	3.1	----	----
Unknown	Test	23.9	17.4	6.5	3.4	0.089 x 10000 = 890
Unknown	Test	27.5	17.7	9.8	6.7	0.0096 x 10000 = 96

[1] Calib, Calibrator.

Table 4. Example ΔC_T Calculations (Amplification factor = 2):

Target Cells in Sample	Sample Type	$C_{T,target}$	ΔC_T	CCE in Test Sample ($2^{-\Delta CT}$ x cells in Calib[1])
10000	Calib[1]	21.4	__	__
Unknown	Test	23.9	2.5	0.177 x 10000 = 1767
Unknown	Test	27.5	6.1	0.014 x 10000 = 146

[1] Calib, Calibrator.

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